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14. ABSTRACT

Integrin alpha 11 (Itga11) is the most recently identified integrin subunit and may be required for the activation of fibroblasts during wound healing and fibrotic disease. To investigate the role of Itga11 in fibroblast behavior we are using cells derived from mice that are genetically null for this gene. We have found that fibroblasts lacking Itga11 (KO) repair a scratch in a confluent monolayer faster than their normal (Het) counterparts, and that this can in part be explained by increased proliferation along the margins of the scratch in the KO cultures. When cultured in collagen gels fibroblasts remodel the gel and alter their expression of genes such as Col1A1 and αSMA, markers of activated fibroblasts. Despite the fact that previous experiments showed that KO cells are deficient in gel contraction, we have found no difference in the expression of Col1A1 or αSMA in the KO cells. Preliminary data suggest that expression of MMP13, a major collagenase, may be decreased in KO cells. Thus to date, the differences between Het and KO fibroblasts are subtle.

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Introduction

Scleroderma is characterized by fibrosis, the replacement of healthy tissue with collagenous matrix. The collagen-binding integrins are key regulators of collagen production. The most recently described integrin alpha subunit, integrin alpha 11 (Itga11) is normally expressed in fibroblasts and in areas of tensile stress, and is over-expressed in fibrotic tissues. We hypothesize that Itga11 expression is elevated in activated fibroblasts resulting in signaling events that contribute to increased synthesis and deposition of collagen. To better understand the role of Itga11 *in vivo* we are using Itga11 null mice. Preliminary data showed that explanted dermal fibroblasts from the Itga11 null mice were deficient in contraction of collagen lattices, an *in vitro* model of force transduction. We are using this model to further investigate the role of Itga11 in collagen production and breakdown, force transduction, cell proliferation and apoptosis, processes that are abnormal in fibrosis. In addition, we are using an *in vitro* 'scratch' model where to investigate the repopulation of a denuded area of a confluent culture of fibroblasts. Fibroblast behavior during wound healing is similar to that observed in fibrosis. Therefore, we will investigate the role of Itga11 *in vivo* by comparing the healing of cutaneous wounds in wild type and Itga11 null mice. The data generated by the experiments detailed in this proposal will describe the mechanism of action of Itga11 and determine its role in the etiology of fibrosis and scleroderma.

Body

The heterozygous (Het) phenotype of one intact Itga11 allele and one null allele appears indistinguishable from the wild-type (WT). Due to the ongoing problems with breeding the Itga11 null (KO) mice, and small litter sizes on the C57BL/6 genetic background, we have maintained breeding pairs as Het paired with KO, thus only generating Het and KO littermates. It is important that primary cells are derived from mice of the same sex, and our previous experience has shown that cells isolated from female mice grow faster and survive longer in culture than cells derived from male mice. We have chosen to use cells derived from female mice and to compare Het and KO genotypes. While it would be preferable to compare WT and KO, breeding problems have made this impossible.

In vitro studies in two-dimensional culture.

Integrins have important roles in cell proliferation and migration during wound healing. We determined if the absence of Itga11 affected the rate of proliferation in isolated dermal fibroblasts. Equal numbers of Het and KO cells were seeded into 96 wells and the number of cells present measured after 24, 48 and 72 hours in culture. Several assays are available to measure cell numbers, we chose to use the Cyquant fluorescent assay from Invitrogen. Cyquant dye fluoresces strongly when bound to double stranded DNA and the fluorescence is linear over a large range of cell numbers. Using this assay we have not observed any difference in the overall rate of proliferation between Het and KO fibroblasts (figure 1).

We have used an *in vitro* 'scratch' assay to investigate the role of Itga11 in proliferation and migration. Primary dermal fibroblasts isolated from Het and KO mice were cultured to confluence on chamber slides. Using a sterile pipet tip the confluent monolayer of cells was scratched to create an *in vitro* model of a cutaneous wound. The scratches were photographed immediately and after 24 and 48 hours to monitor the rate at which the scratch was repopulated. Figure 2 shows images from a representative experiment. At 24 hours the cells at the edge of the scratch have started to migrate into the empty area (panels C and D), although the area of the scratch contains fewer cells in the Het cultures than the KO. After 48 hours cultures of KO cells have entirely filled in the scratched area (panel F). In contrast, the scratched area in the Het cells is still clearly visible at 48 hours.

Repopulating the denuded area involves both migration and proliferation. Figure 1 shows that in standard cultures of cells we do not observe differences in the overall rate of proliferation between Het and KO cells. However cellular responses could be different along the margins of the scratch. We determined the rate of cell proliferation along the edges of the scratch using the thymidine analog 5-bromo-2-deoxyuridine (BrdU). BrdU is incorporated into DNA as it is being replicated, thus it is present only in cells that have recently, or are currently, undergoing mitosis. BrdU was added to the cultures for the final 4 hours of incubation and positive nuclei visualized using a BrdU specific antibody. The number of BrdU positive nuclei and the total number of nuclei (labeled with DAPI (4',6-diamidino-2-phenylindole)) were counted in 6 fields along the edge of the scratch, and the number of BrdU positive nuclei expressed as a percentage of the total nuclei in each field. Figure 3 shows that the percentage of nuclei positive for BrdU is higher in the KO cultures than the Het cultures. Taken together with the pictures in figure 2, these data show that Itga11 null fibroblasts can repopulate a denuded area faster than normal fibroblasts, and that at least a part of this effect can be explained by the fact that, compared with the Het cultures, a higher percentage of the KO cells adjacent to the scratch are actively dividing.

<u>*In vitro* studies in three-dimensional culture.</u>

In vivo, fibroblasts exist in a three-dimensional matrix with few direct cell contacts. To more closely replicate these conditions *in vitro* fibroblasts can be cultured in gels of collagen type I. In floating (unstressed) collagen gels fibroblasts remodel and contract the initially loose matrix of collagen fibrils into a dense tissue like structure. We have found that KO fibroblasts are deficient in their ability to contract collagen gels even when stimulated with the profibrotic cytokine TGFβ. We have cultured fibroblasts on plastic and in collagen gels, with and without TGFβ, to determine the effects of deleting Itga11 on the expression patterns of genes involved in wound healing and fibrosis.

For two dimensional culture on plastic, cells were seeded into 6-well plates at a density of 5×10^4 cells per well. For cells cultured in collagen gels, fibroblasts were suspended in a solution of 1.5mg/ml bovine collagen type I in DMEM at a density of 1×10^6 cells/ml. 0.5 ml of cell suspension were aliquoted into BSA coated 24-well plates and incubated for 1 hour at 37°C to form gels. 1 ml medium was added to gels which were gently nudged to ensure that they were floating freely. In both the two and three-dimensional systems

recombinant human TGFβ (R&D Systems) was tested at a final concentration of 10 ng/ml. Total RNA was extracted from cell cultures and cDNA was synthesized from 0.5μg total RNA for each sample. Expression of Itga11, collagen type I, αSMA, MMP13 and Rpl13 were quantified by quantitative reverse transcriptase PCR (QRT-PCR) using a Stratagene MX300P system and Sybr green detection (Sybr fastmix, Quanta Biosciences). Expression of each gene of interest was normalized to Rpl13 as a control, and compared to the expression observed in the Het cells cultured on plastic (two-dimensional) in the absence of TGFβ.

Previous data showed that Itga11 was overexpressed in fibrotic tissues. Therefore we first tested whether expression of Itga11 could be increased by the addition of TGFβ to fibroblasts *in vitro*. Expression analysis by QRT-PCR showed that when cultured on plastic or in collagen gels, addition of TGFβ resulted in increased Itga11 expression compared to the untreated cells (Figure 4). The degree of stimulation varied significantly between different isolates of cells, and between different individual experiments, indicated by large error bars. Thus, while we are confident that TGFβ causes increased Itga11 expression in fibroblasts *in vitro*, we are unable to draw conclusions about the effects of culture system on the expression of Itga11.

Fibrotic conditions are characterized by replacement of healthy tissue by collagen type I produced by activated fibroblasts. We determined expression of collagen type I alpha 1 chain (Col1A1) in mouse primary dermal fibroblasts isolated from Het and KO mice, in two and three-dimensional culture systems in the presence and absence of TGFβ. Over multiple experiments we did not observe increased expression of collagen type I when TGFβ was added to fibroblasts cultured on plastic in either the Het or KO fibroblasts (Figure 5, black bars). When fibroblasts were cultured in collagen gels we found that addition of TGFβ did result in increased expression of collagen type I, and that there was no appreciable difference in the degree of stimulation between the Het and KO cells (Figure 5, red bars). The data presented in figure 5 are fold change in expression normalized to the expression in the untreated Het cells in the two different culture systems.

In addition to elevated expression of collagen type I, the expression of α smooth muscle actin (αSMA) is currently the most accepted marker of activated fibroblasts. The expression of αSMA allows activated fibroblasts to be contractile which contributes to contraction in healing wounds and fibrosis. Itga11 KO fibroblasts are deficient in their ability to contract collagen gels. Therefore we compared the expression of αSMA in KO and Het fibroblasts cultured on plastic and in collagen gels. Figure 6 shows that αSMA expression is the same in KO and Het fibroblast and is increased to the same degree in response to addition of TGFβ, both on plastic and in collagen gels. Again we observed a great degree of variability between the two sets of cell isolates tested. The degree of stimulation of αSMA expression appears slightly higher when the fibroblasts are cultured in the collagen gels (Figure 6, compare red bars with black bars) but this is not a statistically significant result based on current data.

The accumulation of collagen depends on the balance of collagen production and breakdown. Likewise, the ability of fibroblasts to contract collagen gels requires both ability of fibroblasts to contract by expression of αSMA and the breakdown of components of the extracellular matrix (ECM). MMP13 is the major proteinase for collagen type I. Because we did not observe changes in Col1A1 or αSMA expression, we next examined expression of MMP13. Figure 7 shows that in the single experiment we have carried out so far, expression of MMP13 appears to be lower in KO fibroblasts compared to the Het in cultures on plastic not exposed to TGFβ (figure 7 black bars, compare Het and KO). In the collagen gels, MMP13 expression in the KO fibroblasts is lower than that observed in the Het cells both in the presence and absence of TGFβ. These data are preliminary, but if replicated could explain the lower ability of KO cells to contract collagen gels. However, this result would suggest that an increase in Itga11 expression should result in increased MMP13 expression, which is counter to what one would expect in a fibrotic situation.

Key research accomplishments

- *In vitro* experiments have been carried out using Het and KO fibroblasts isolated from two different paired sets of mice.
- we have not observed differences in the overall rate of proliferation between the het and KO fibroblasts
- KO cells repopulate a scratched area faster than the Het fibroblasts
- This can be partly explained by higher rates of cell proliferation observed along the margins of the scratch in the KO cultures.
- We have used the floating collagen gel system to culture fibroblasts in an environment that is closer to the *in vivo* conditions
- In this three-dimensional system we have found that the absence of Itga11 expression does not result in any difference in expression of collagen type I and αSMA, two genes that are overexpressed during wound healing and fibrosis
- Preliminary data suggest that MMP13 may be expressed at lower levels in the KO cells. This could explain the defect in contraction of collagen gels.

Reportable outcomes

There are currently no reportable outcomes.

Conclusion

This year we have made good progress on the *in vitro* aim of this project, and have completed Task 1. Unfortunately breeding of the mice has remained slow and we have only been able to complete half of Task 2: the analysis of gene expression in floating collagen gels using quantitative RT-PCR. In addition we have only been able to perform one small wound healing study that we are currently analyzing.

C57BL/6 mice are generally poor breeders and it can take several pregnancies to get a litter that survives too weaning. In addition litter sizes are generally small, typically 4-6

pups in total. To encourage successful breeding males have been removed from the cage when females are pregnant, and breeding females have been supplied with enriched breeder chow. Once a female has given birth, even if the pups did not survive, the females are mated again to ensure multiple pregnancies, which usually leads to increased survival of the subsequent litters. Despite these measures, many females that got pregnant did not care for their litters and few pups have survived to weaning. This has meant that the supply of mice for experiments has been very limited. Because of the problems with breeding, we propose to focus on *in vitro* studies for the final year of this award. In the original application we proposed crossing the Itga11 null mice with a transgenic mouse in which expression of a mutated TGFβ receptor in fibroblasts results in skin and lung fibrosis. With the problems that we have had with breeding it is not feasible to generate the genotypes required for the proposed analysis. In addition it is unlikely that we will generate sufficient animals to perform the proposed wound healing studies. Therefore we propose the following revised statement of work:

Task 1. Studies in two-dimensional cell culture. Further investigate the faster scratch healing by the KO fibroblasts by examining the rate of apoptosis in Het and KO cultures. Use a transwell migration assay to examine the rates of migration in Het and KO fibroblasts.

Task 2. Use floating collagen gel contraction assays to assess the differentiation of fibroblasts by immunofluorescent staining to confirm QRT PCR data for α–SMA, collagen type I, and MMP-13 expression. Repeat analysis of MMP13 by QRT-PCR to determine if differences in expression between the Het and KO fibroblasts are real. If we find that MMP13 expression in altered in KO fibroblasts we will extend the studies to examine the activity of MMP13 using zymography. We will also determine the rate of apoptosis in Het and KO fibroblasts cultured in collagen gels using the TUNEL assay and immunofluorescence for cleaved caspase 3, a marker of apoptosis.

Task 3. Determine differences in cell behavior in attached collagen lattices where cells experience greater tensile stresses than those in the floating collagen gels. The same assays as described in task 3 will be used.
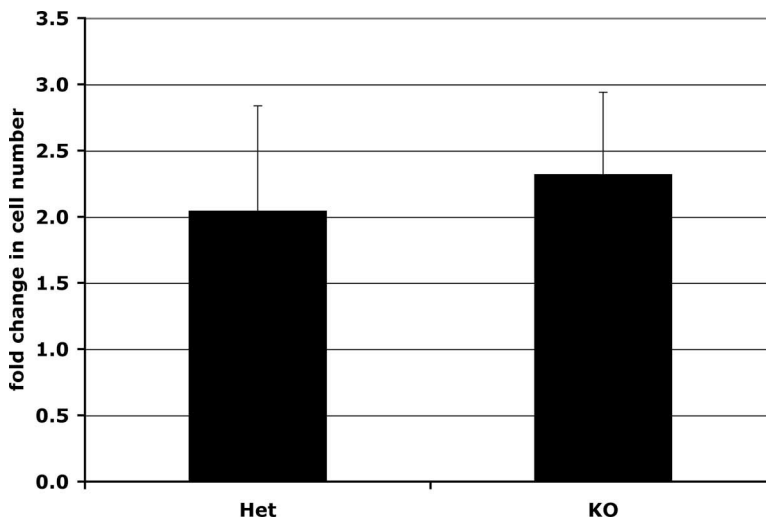
Appendix 1.

Figure 1. Under standard culture conditions there are no obvious differences in proliferation rate between Het and KO fibroblasts. Het and KO fibroblasts were seeded into 96 wells at $1.5x10^3$ cells per well in 100 µl of DMEM plus 10% FBS. Cell numbers were measured after 24, 24 and 72 hours using the Cyquant assay (Invitrogen). Cyquant dye fluoresces strongly when bound to double stranded DNA and therefore gives an accurate representation of the number of nuclei present in each well. Medium was aspirated and dye added in Hanks Buffered Salt Solution (HBSS). Cells were incubated with the dye for 30 minutes before fluorescence was measured using a SpectraMax plate reader with excitation at 485nm and emission at 530nm. Data presented are the mean and standard deviation of the fold change in cell number between 24 and 48 hours for three Het and three KO cell isolates.
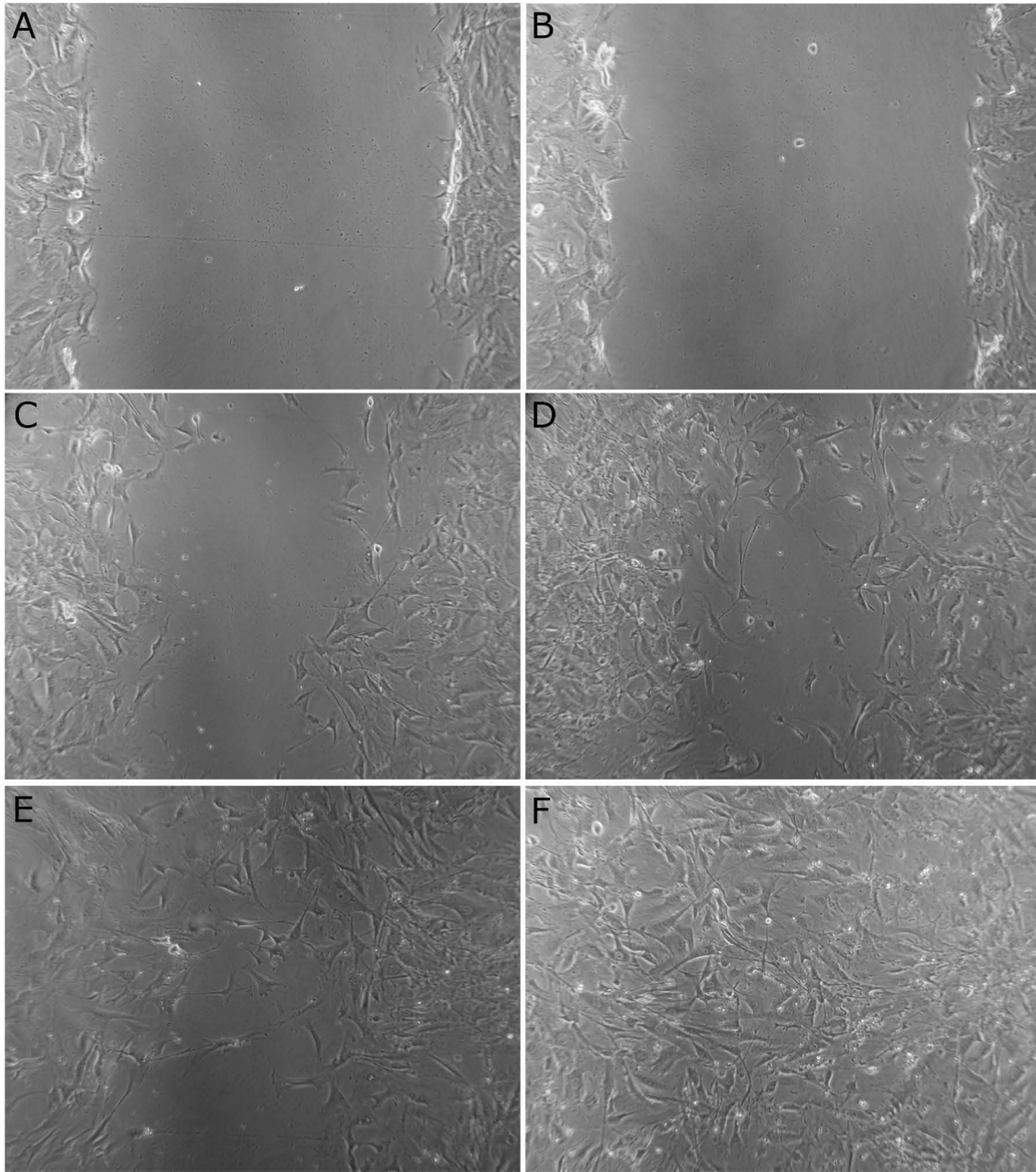
Figure 2. Itga11 null fibroblasts repopulate a scratch faster than Het cells. Primary dermal fibroblasts isolated from Het (A, C, E) or KO (B, D, F) mice were seeded into 4-well chamber slides at a density of $5x10^4$ cells/well in a volume of 1 ml DMEM containing 10% FBS. The following day a sterile pipet tip was used to make two scratches across the well perpendicular to each other forming a cross in the confluent cell layer. After forming the scratch the medium as aspirated, the monolayer gently washed with fresh medium and 1ml fresh serum containing medium was added. The area of the scratch just above the cross was photographed immediately (A, B) after 24 hours (C, D) and 48 hours (E, F) so that the area being photographed was close to the same at the different time points. Pictures are from a single experiment that is representative of three independent experiments.
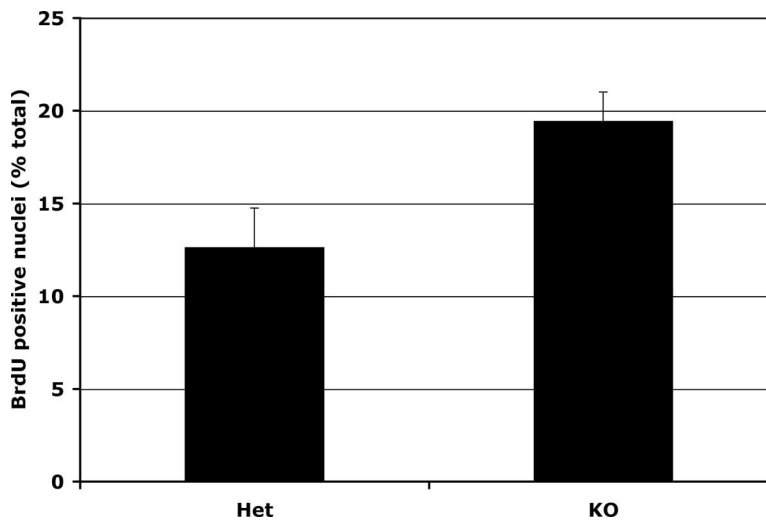
Figure 3. **Itga11 null fibroblasts adjacent to the scratch proliferate faster than Het cells**. 10μM BrdU was added to the cultures for the final 4 hours of incubation. Cells were fixed with 3% paraformaldehyde followed by incubation with a BrdU specific antibody (Bioegend, clone MoBU-1 mouse IgG1) and a secondary anti-mouse antibody conjugated with Alexa-594. To label all nuclei 2 μg/ml 4',6-diamidino-2-phenylindole (DAPI) was added with the secondary antibody. Samples were mounted with an a aqueous fluorescent mounting medium and photographed with a Nikon Eclipse E 800-S fluorescence microscope. The number of BrdU positive nuclei (red) and the total number of nuclei (blue) were counted in 6 fields along the edge of the scratch, and the number of BrdU positive nuclei was expressed as a percentage of the total nuclei in each field. Data presented are the mean and standard deviation from three independent experiments.
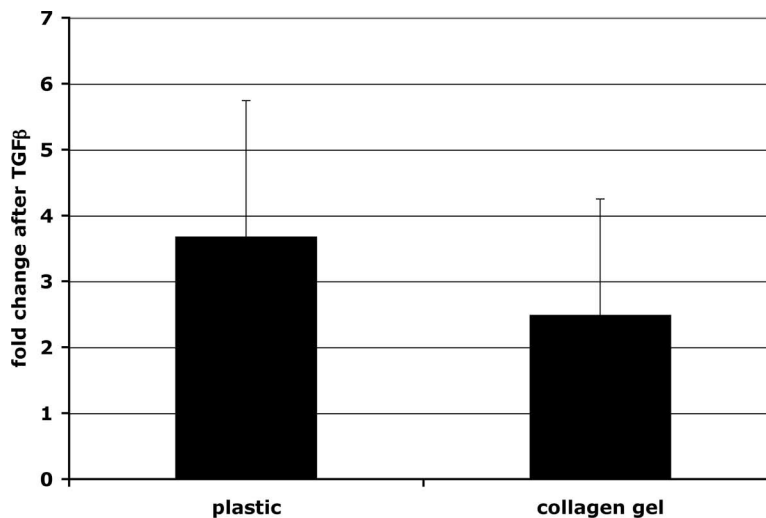

Figure 4. Itga11 expression increases in response to TGFβ treatment *in vitro*. Het fibroblasts were cultured on plastic or in collagen gels for 48 hours in the presence or absence of 10 ng/ml TGFβ. Total RNA was isolated using mini spin columns (Omega

Biotek) and 0.5µg RNA used to synthesize cDNA (cDNA syntheis kit, Quanta Biosciences). QRT-PCR reactions were carried out using Sybr green detection (Sybr Fastmix, Quanta Biosciences) using a Stratagene MX300P system. Expression of Itga11 was normalized to the expression of a control gene, Rpl13, in each sample. Primer sequences used were: Itga11 forward: TGAACACTGTGTCCCTGACC, Itga11 reverse: CGCGTGCTCTCTATGATGAA, Rpl13 forward: TTTTGCCAGTCTCCGAAT, Rpl13 reverse: TGCTTTATGGAAAATTTATTGC. For each individual experiment the fold change in Itga11 expression after TGFβ treatment was calculated and data presented are the mean and standard deviation of 4 independent experiments.
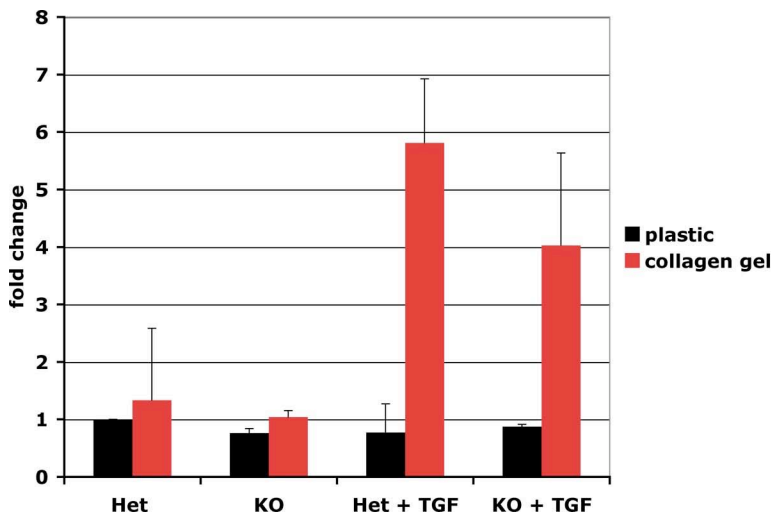

Figure 5. Expression of type I collagen is normal in Itga11 null fibroblasts. Itga11 null (KO) and Het fibroblasts were cultured on plastic or in collagen gels for 48 hours in the presence or absence of 10 ng/ml TGFβ. RNA was extracted, cDNA synthezised and QRT-PCR reactions performed as described in the legend to figure 3. Primer sequences for the alpha1 chain of type I collagen were forward: GTATTGCTGGACAACGTGGT and reverse: AATGCCTCTGTCACCTTGTTC. Expression of type I collagen was normalized to expression of Rpl13 in each sample. For each experimental condition (culture on plastic (black bars) and in collagen gels (red bars)) the normalized expression of typeI collagen was compared to the expression in the Het fibroblasts untreated with TGFβ. Data presented are the mean and standard deviation of 2 independent experiments.
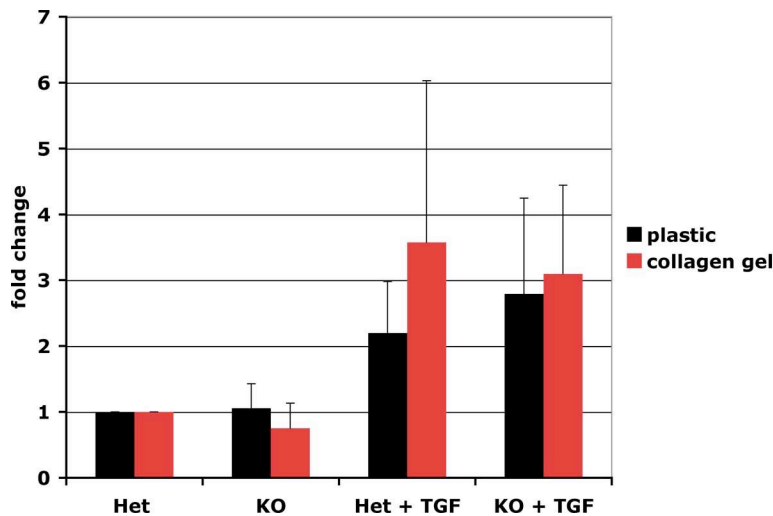
Figure 6. Expression of αSMA is normal in Itga11 null fibroblasts. αSMA expression was analyzed in the same samples described in figure 4. Primer sequences for αSMA were forward: ATGTGTGAAGAGGAAGACAGCA, and reverse: GCCGTGTTCTATCGGATACTTC. Data presented are the mean and standard deviation of 2 independent experiments.
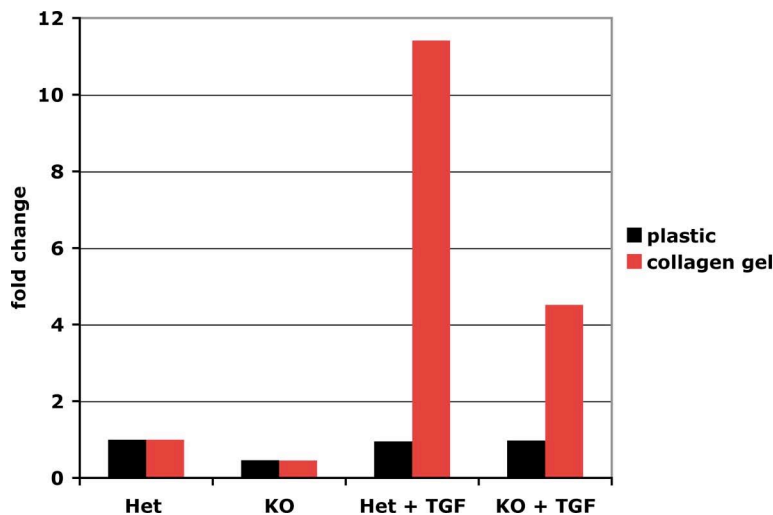

Figure 7. Expression of type I collagen is normal in Itga11 null fibroblasts. MMP13 expression was analyzed in samples prepared as described in figure 4. Primer sequences for MMP13 were forward: CATGAGAAAACCAAGATGTGGA and reverse: TCTGGTGAAATTCAGTGGTGTC. Expression of type I collagen was normalized to expression of Rpl13 in each sample. Data presented are from a single experiment.